DRUG-INDUCED HEMOLYSIS OF G6PD-DEFICIENT HUMAN ERYTHROCYTES IN RAT HOSTS

DONALD STEVEN BAIM

1975

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INTRODUCTION

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymatic defect of the human erythrocyte. More than 100 million individuals are affected, and over 100 enzymatic variants have been described (31). Although possible associations between G6PD deficiency and various systemic diseases have been suggested, the main morbid effect of G6PD deficiency is an increased sensitivity to hemolysis by a wide range of drugs and febrile illnesses (2). This sensitivity to drug-induced hemolysis is not uniform among G6PD-deficient individuals: individuals with one variant form of the enzyme may be particularly sensitive to hemolysis by a particular drug, while individuals with another variant form may be resistant (30). Adequate prediction of drug-induced hemolysis therefore requires knowledge of the sensitivity of each G6PD variant to different, potentially hemolytic drugs. Such knowledge has not been provided by current methods of studying drug-induced hemolysis: clinical reports of hemolysis, trial administration of hemolytic drugs to known G6PD-deficient individuals, and transfusion of radioactivaly tagged G6PD-deficient erythrocytes into drug-taking normal human recipients, have been neither safe nor reliable means of study; measurement of alterations in the morphology, biochemistry, and fragility of erythrocytes exposed *in vitro* to hemolytic drugs or their metabolites, have not been reliable predictors of *in vivo* hemolysis. Moreover, there is no reliable means of evaluating new drugs for their potential to hemolyze G6PD-deficient erythrocytes prior to their clinical introduction.

The purpose of this research is to test the susceptibility to drug-induced hemolysis of human G6PD-deficient erythrocytes in the circulation of specially prepared rats. These rats are injected with an emulsion of ethyl palmitate to block reticuloendothelial function, and a purified anticomplementary factor from the venom of the cobra, *Naja naja* (9), to inhibit complement mediated hemolysis of the human erythrocytes. This preparation, first employed in 1970 (8,48), extends the half-life of transfused erythrocytes from 5-10 minutes, to 24 hours. The mechanism of reticuloendothelial blockade, and the detailed erythrokinetics of this

model have been studied previously (19,23,37). Castro, Finch, and Orlin have employed this model to study the effects of oxygen tension and anti-sickling agents on the survival of human sickle erythrocytes, with promising results.(7,18,33,6). We now study the drug-induced hemolysis of G6PD-deficient erythrocytes by administering several drugs known to cause hemolysis of G6PD-deficient erythrocytes, to rats which have been prepared in the manner described above, and transfused with erythrocytes from G6PD-deficient and normal individuals. The three drugs - acetylphenylhydrazine, nitrofurantoin, and primaquine - were administered parenterally to the rats for two days prior to transfusion, that high blood levels of drug metabolites might be induced. Erythrocytes from six normal and four G6PD-deficient humans were labeled with 51 -chromium and transfused into rats treated either with 5% dextrose in water or one of the hemolytic drugs. Drug-induced hemolysis was measured by comparing the 51-Cr erythrocyte survivals in dextrose- and drug- treated rats, during the 24 hours after transfusion.

The results of these experiments, and a review of current knowledge of drug-induced hemolysis in G6PD deficiency, are presented.

MATERIALS AND METHODS

Animal recipients

Animal recipients of human blood transfusions were male Sprague-Dawley rats (Charles River Breeders, Boston) weighing between 225 and 300 grams.

Two hours prior to transfusion, each animal was given ethyl palmitate 0.5 gm/kg, and cobra venom anticomplement factor, 10 units, by tail vein injection under light ether anaesthesia. This material was prepared by homogenization of 7.5 ml of ethyl palmitate (Eastman Kodak, Rochester) with 17.5 ml of 5% dextrose in water and 0.2 ml of Tween 20. Sonication for three minutes (Bronson Sonifier) yielded an emulsion containing 0.5 gm of ethyl palmitate per 2 ml of emulsion.. Immediately prior to injection, the appropriate amount of emulsion was mixed with 10 units of cobra venom factor (Cordis Labs , Miami) diluted in 1 ml of normal saline.

Human subjects

Because of the sex-linked nature of G6PD-deficiency, only male subjects were studied. Of the ten subjects studied, four were G6PD-deficient and six were normal, as determined by quantitative G6PD assay. Hematocrit, reticulocyte count, and hemoglobin electrophoresis were obtained as indicated in Table 1. All subjects denied previous exposure to major hemolytic drugs, and only DdL (Mediterranean G6PD-deficient male) gave a clinical history compatible with prior hemolysis (see footnote to Table 1).

Hemolytic drugs

Three drugs known to cause hemolysis in patients with G6PD deficiency were compared with 5% dextrose in water for their effect on human erythrocytes in the rat circulation. Rats were injected with drug for one to two days prior to transfusion to allow accumulation of drug metabolites, since the metabolites are known to be more active in producing hemolysis than the drugs themselves (39). Drug doses were selected empirically during preliminary experiments for their ability to observably decrease the 24-hour survival of black G6PD-deficient erythrocytes in rat hosts, without affecting normal erythrocytes. In general these doses are an order of magnitude larger than accepted mg/kg human theraputic doses.

Acetylphenylhydrazine is an extremely potent hemolytic drug formerly used in the treatment of polycythemia vera. It is toxic to both normal and G6PD-deficient erythrocytes, and has been used in studies of drug-induced hemolysis (1). A dose of 15 mg/kg was dissolved in 0.9 ml of 5 % dextrose in water, and 0.1 ml of 95% ethanol was added to facilitate solution. This dose was administered sub-cutaneously 24 hours prior to transfusion, and intraperitoneally at the time of transfusion.

Sodium nitrofurantoin is a commonly used urinary antiseptic capable of producing clinically significant hemolysis in G6PD-deficient subjects (2). Parenteral nitrofurantoin (Furadantin, Eaton Labs) was administered in a dose of 30 mg/kg in 1 ml of 5% dextrose in water, given sub-cutaneously 24 and 48 hours prior to transfusion, and intraperitoneally at the time of transfusion.

Primaquine phosphate is an 8-aminoquinolone antimalarial agent which has played a central role in the elucidation of drug-induced hemolysis in G6PD deficiency. It was obtained in a chemically pure form from Winthrop Labs, and administered in the dose and manner described above for nitrofurantoin.

Experimental procedure

Twelve ml of blood was drawn from each human subject within the 24 hours prior to transfusion, and anticoagulated with 3 ml of acid-citrate-dextrose solution in commercially prepared, evacuated test tubes (Vacutainers No. 4786, Becton-Dickenson). Blood was stored at 4°C until use. Prior to transfusion, 40 uCi of 51-Cr (sodium chromate, New England Nuclear, Boston) was added per ml of blood, and this mixture was incubated at 37°C for 30 minutes with frequent mixing. Labeled blood was washed twice in equal volumes of normal saline, and resuspended in saline to a final volume of 10 ml. One ml of this blood was transfused into each of eight rats via the dorsal vein of the penis, so as to avoid radioactive contamination of the tail. At the same time, these rats received their final doses of hemolytic drug (see below).

Following transfusion, animals were allowed food and water *ad lib*. Hourly for the first six hours, and at 12, 18 and 24 hours after transfusion, 20 microliter blood samples were obtained from

the cut surface of the tail using 20 microliter Microcaps (Drummond Scientific). Each Microcap was placed in a small plastic tube and counted for one minute in a well-type scintillation counter. One minute counts in the range of 4000 counts were obtained, corresponding to random counting errors of 2 percent. Periodic microhematocrits were unchanged during the course of the experiment. No significant extra-erythrocytic 51-Cr activity could be detected in the supernatant rat plasma obtained during the microhematocrit determinations. Since previous studies (33) have shown elution of radioactive label and hemoconcentrative errors of tail-vein sampling to be insignificant, these parameters were not explored.

The experimental procedure is presented in tabular form below:

		DOSAGE SCHEDULE			PREP.	TRANSFUSE
RAT NO.	DRUG+DOSE	-48hr	-24hr	0hr	-2hr	0hr
1,2	D5W	SC	SC	IP	*	1ml
3,4	APH 15mg/kg	-	SC	IP	*	1ml
5,6	FUR 30mg/kg	SC	SC	IP	*	1ml
7,8	PRI 30mg/kg	SC	SC	IP	*	1ml

SC = sub-cutaneously
IP = intraperitoneally
* = ethyl palmitate 0.5gm/kg, plus cobra venom 10 units
ALL TIMES ARE GIVEN RELATIVE TO 0-HR (TIME OF TRANSFUSION).

RESULTS

Survival of transfused erythrocytes

Baseline 51-Cr activity was determined for each rat as the mean 51-Cr activity in 20 microliter tail vein blood samples drawn 1, 2, and 3 hours after transfusion. Erythrocyte survival was then calculated as the ratio of 51-Cr activity in the 6, 12, 18, and 24 hour post-transfusion blood samples, to the baseline activity. Survival curves were plotted for each human subject, using the mean survival in duplicate rats. Representative curves for a normal, black G6PD-deficient, and Mediterranean G6PD-deficient individual are shown in Figures 1, 2, and 3 respectively. Separate curves are shown for rats treated with 5% dextrose (control), and for each of the three hemolytic drugs. In each case, the error bars indicate the range of the duplicate rats.

Erythrocytes from all three individuals had comparable survival (50-60 percent 24-hour survival) in rats treated with 5% dextrose alone. In all cases, administration of a hemolytic drug to the rat recipients reduced the 24-hour survival of the transfused erythrocytes. This reduction was least pronounced with erythrocytes the normal subject, and most pronounced with erythrocytes from the Mediterranean G6PD-deficient subject. Erythrocytes from the black G6PD-deficient subject showed an intermediate reduction in survival. In each case the reduction is most manifest at 24 hours after transfusion, though some early reduction is seen with erythrocytes from the Mediterranean subject. For this reason, subsequent calculations were based on 24-hour erythrocyte survivals as shown in Table 2.

Measurement of drug-induced hemolysis

For each donor, drug-induced hemolysis is manifest as a decreased erythrocyte survival in drug-treated animals relative to 5% dextrose-treated animals. This decrease has been quantitated as:

$$\text{PERCENT DRUG-INDUCED HEMOLYSIS} = 100\% \text{ MINUS } \left(\frac{\text{SURVIVAL (drug)}}{\text{SURVIVAL (D5W)}} \times 1\right)$$

so as to correct for the non-specific removal of human erythrocytes as observed in the dextrose-treated control rats. Using this form-

ulation, the drug-induced hemolysis for each human subject and each hemolytic drug is given in Table 3, and in Figure 4.

Some drug-induced hemolysis of normal erythrocytes was observed, with means of 10 to 17 percent hemolysis, depending on the particular drug. G6PD-deficient erythrocytes had mean drug-induced hemolysis 2.5 to 3 times larger than did the normal erythrocytes. Among the G6PD-deficient erythrocytes, hemolysis was most pronounced with erythrocytes from the Mediterranean subject - some 4 to 7 times that of normal erythrocytes. The difference in drug-induced hemolysis between normal and G6PD-deficient erythrocyes is statistically significant for each of the three drugs tested, as determined by the Wilcoxon Rank Sum Test (47). These results are summarized below, with the percent drug-induced hemolysis for the Mediterranean G6PD-deficient erythrocytes given in parentheses:

PERCENT DRUG-INDUCED HEMOLYSIS 24 HOURS AFTER TRANSFUSION

	MEAN OF 6 NORMALS	MEAN OF 4 G6PD-DEFIC	STATISTICAL SIGNIF. OF NORMAL vs. G6PD-DEFIC.
PRIMAQUINE	17%	53% (70%)	$p < 0.01$
NITROFURA.	10%	31% (74%)	$p < 0.03$
ACETYLPHEN.	17%	42% (68%)	$p < 0.01$

DISCUSSION

This discussion is divided into two main parts. Part I is a review of current knowledge concerning G6PD deficiency, dealing with the history, enzymology, and consequences of G6PD deficiency. Part II deals with the experimental results described above, and with their implications for the study of drug-induced hemolysis.

PART I - G6PD Deficiency and Drug-induced Hemolysis

Antimalarial drugs, especially primaquine and other 8-aminoquinolines have long been known to induce hemolytic anemia in a susceptible fraction of blacks. Hemolysis of tagged erythrocytes from primaquine-sensitive individuals occurred after transfusion into normal subjects to whom primaquine was administered, establishing the intrinsic nature of the erythrocytic defect in primaquine sensitivity. Using this method, primaquine sensitivity was shown to be but one manifestation of sensitivity to many drugs including sulfonamides, phenacetin and nitrofurantoin (12). Biochemical studies of sensitive erythrocytes revealed a decreased level of reduced glutathione (GSH), further decreased by exposure to hemolytic drugs. Investigation of the hexose monophosphate shunt, by which the erythrocyte reduces glutathione, revealed that G6PD activity was decreased in primaquine-sensitive erythrocytes.

Glucose 6-phosphate dehydrogenase

Approximately ten percent of red cell glucose metabolism proceeds via the hexose monophosphate shunt, the sole erythrocytic pathway for the formation of the reduced triphosphopyridine nucleotide, NADPH. NADPH is necessary for the reduction of oxidized glutathione (GSSG) to its reduced form (GSH), and for the defense of sulfhydryl groups in hemolglobin and other red cell constituents from oxidation. Reduced glutathione, in turn, is necessary for the detoxification of hydrogen peroxide by the enzyme, glutathione peroxidase.

G6PD controls the entry of glucose into the hexose monophosphate shunt, shown in Figure 5 along with the rest of red cell glucose metabolism. G6PD converts glucose 6-phosphate to 6-phospho-gluconolactone (not shown), while reducing one molecule

of NADP to NADPH. The lactone is subsequently converted to 6-phosphogluconate. This in turn forms pentose phosphate with conversion of a second molecule of NADP to NADPH and release of carbon dioxide. In summary, one molecule of glucose processed by the hexose monophosphate shunt allows the conversion of two molecules of NADP to NADPH.

In its active form, human G6PD is a dimer with a molecular weight of approximately 100,000 daltons (49). Its structure is determined by a gene locus on the X chromosome. Males and homozygous females therefore have a single type of G6PD, while heterozygous females are G6PD-mosaics created by random inactivation of X chromosomes in the bone marrow stem cells. More than one hundred variants of G6PD have been cataloged (2,30,31,45). They are characterized by their biochemical properties: affinity for substrate and substrate analogues, pH optima, thermal stability, and electrophoretic mobility at pH 8.6 (3). More than 100 million individuals are estimated to harbor variant forms of G6PD (31), the frequencies of which are subject to marked racial and geographical differences.

The most common, or wild type, of G6PD is known as B+, and is found in more than 99 percent of the white population. G6PD Mediterranean, or B-, is found with significant (more than one percent of males) frequency among Mediterraneans, Sardinians, Sephardic Jews, and in as many as 50 percent of Kurdish Jews. It is associated with a decreased number of biochemically abnormal enzyme molecules, and with marked impairment of red cell G6PD activity. Other enzyme variants are far less common among whites.

The B+ enzyme is also found in 70 percent of black males, but 20 percent carry the A+ variant, which has greater electrophoretic mobility at pH 8.6, and a 20 percent reduction in enzymatic activity as measured by Vmax. An additional 10-14 percent of black males carry the A- (black G6PD deficiency) form of G6PD, whose decreased stability leads to impaired G6PD activity in older erythrocytes (31).

While typing the precise G6PD variant is limited to a few specialized laboratories, G6PD deficiency itself can usually be detected by simple screening procedures (2,15,45). Confirmation

requires quantitative G6PD assay in which the rate of substrate conversion is measured under optimal conditions. This allows calculation of the enzymatic Vmax, which in most cases corresponds to the erythrocytic G6PD activity. Some cases have been reported, however, of enzymes with relatively normal V max but markedly reduced erythrocytic G6PD activity, owing to excessive inhibition of the variant G6PD molecule by NADPH and ATP present in the red cell (49,50).

Diagnosis of G6PD deficiency during or after an episode of hemolysis is easily made in Mediterranean G6PD deficiency, but may be difficult in black G6PD deficiency since the younger erythrocytes which survive hemolysis in blacks may have relatively normal enzyme levels. High speed centrifugation allows assay of G6PD activity in the oldest and most dense remaining erythrocytes, and may be used establish the diagnosis of black G6PD deficiency in the face of hemolysis. The methemoglobin elution test, a cell-by-cell stain reflecting G6PD activity, can also be used to demonstrate remaining G6PD-deficient cells. Low or normal G6PD levels in the face of reticulocytosis following hemolysis is also an indicator of G6PD deficiency. When the diagnosis of G6PD deficiency cannot be made, other etiologies of drug-induced hemolysis must be considered (10) including less common abnormalities of the hexose monophosphate shunt, and unstable hemoglobins (30).

Clinical Consequences of G6PD Deficiency

The main clinical consequence of G6PD deficiency is a predisposition to hemolysis by a wide range of drugs, febrile illnesses and metabolic abnormalities, shown in Figure 6 (29). A more extensive list with documentation can be found in the references (2,45). The typical clinical picture of drug-induced hemolysis is the acute onset of hemoglobinuria, falling hematocrit, and red cell Heinz bodies within 1 to 3 days after a hemolytic drug is begun (21). The extent of hemolysis varies with the dose of the offending drug and with the nature of the deficient enzyme: hemolysis is more severe among Mediterranean than black G6PD deficient individuals, since the young black G6PD-deficient erythrocytes constitute a hemolysis-resistant population with

approximately normal G6PD activity. Thus even in the face of continued drug administration, drug-induced hemolysis in blacks is usually a self-limited process of truncated erythrocyte survival compensated by increased erythropoesis. In contrast, the Mediterranean G6PD-deficient individuals with their severely and uniformly depressed G6PD activity, usually undergo dramatic hemolysi sometimes to red cell counts under one million per cubic milimeter. All attempts to predict hemolytic severity on the basis of the biochemical integrity of variant enzymes are not this successful. Thus the Vmax of G6PD Canton is nearly as low as that of G6PD Mediterranean (B-), and yet the Mediterraneans hemolyze when given chloramphenicol, and the Chinese do not.

Although patterns of hemolytic sensitivity can be established for the different G6PD variants, deviations from these patterns are quite common. Thus a small percentage of black G6PD-deficient soldiers given chlorocuine and primaquine for malarial prophylaxis had a much more profound hemolytic reaction than expected. Equally unexpected was the failure of a small percentage of Sardinians with Mediterranean G6PD deficiency to hemolyze when given large doses of primaquine (30,34). In part these deviations may reflect the existence of sub-variant forms of G6PD deficiency with different hemolytic sensitivities than the more common variants, but this has been documented in only a limited number of cases. A more likely explanation is the influence of extra-erythrocytic factors on drug-induced hemolysis, particularly pharmacogenetic differences in drug metabolism or aquired differences in hepatic and renal clearance of drug metabolites. Thus when erythrocytes from the non-hemolyzing Sardinians were transfused into normal primaquine-taking individuals, they hemolyzed as expected. This picture has also been seen in the hemolysis of erythrocytes from a single G6PD-deficient subject in most but not all recipients treated with thiazolesulfone (3).

Other agents may induce hemolysis in G6PD-deficientindividuals. These include febrile illnesses of viral (infectious hepatitis, influenza A, infectious mononucleosis) and bacterial (pneumonia, typhoid fever) etiology (2,30), and diabetic ketoacidosis (2,5, 30). Severe hemolysis has been produced in some G6PD-deficient

individuals with the Mediterranean or Zähringen variety of G6PD by ingestion of the fava bean (Vicia fava). In certain severe forms of G6PD deficiency, hemolysis may take place in the absence of external stresses. While this is a relatively uncommon manifestation of G6PD deficiency, it is extimated to acccunt for 25 percent of thecases of nonspherocytic hemolytic anemia.

There is no specific therapy for drug-induced hemolysis in G6PD deficiency beyond supportive measures (mannitol, fluids) and the discontinuation of the offending drugs. The decision to discontinue a drug is a clinical one, resting on the need for and alternatives to the drug as well as the severity of the hemolysis (2,30). Avoidance of the known hemolytic drugs in Figure 6 is prudent, particularly in individuals with demonstrated hemolytic potential. Spelnectomy has only rarely been of value in the management of G6PD deficiency manifest as nonspherocytic hemolytic anemia (2).

The individual with G6PD deficiency has decreased G6PD activity in other body tissues besides the erythrocyte. The erythrocyte seems particularly sensitive to the consequences of G6PD deficiency for two reasons: first, the erythrocyte does not possess the alternative sources of NADPH (citric-acid cycle, non-specific hexose 6-phosphate dehydrogenase) available to other cells, and second, the erythrocyte is continually exposed to a high redox potential by virtue of its role in oxygen transport (2). Abnormalities in other tissues may occur, however, as suggested by sporadic accounts of an increased prevalence of cataracts (20), abnormal intravenous glucose tolerance test (14), schizophrenia, regional ileitis, and epilepsy (2) in individuals with G6PD deficiency. A decreased incidence of cancer has also been reported in G6PD-deficient individuals (43). Perhaps most significantly, a decreasing incidence of G6PD deficiency with advancing age has been observed, suggesting that G6PD-deficient individuals may have a higher mortality than normal individuals (35,46). This observation has not been confirmed in a more recent and much larger study of 30,000 black men at VA hospitals (32), so that further studies are needed.

The relationship between G6PD deficiency and sickle cell

anemia has been the subject of a recent controversy. Initial reports (36) suggested an increased prevalence of G6PD deficiency in sickle cell disease (33 percent of 15 sickle cell patients were G6PD deficient versus 11 percent of 102 normal blacks). This suggested that G6PD deficiency may ameliorate the effects of sickle cell disease, perhaps by destroying old, irreversibly sickled erythrocytes, while the ongoing sickle-related hemolysis of older cells might decrease the mean red cell age and thereby increase the mean G6PD level. This association was initially challenged (41), but has been confirmed in the most recent study (4). This study, however, showed an increased incidence of G6PD deficiency in both sickle cell and their non-sickle siblings, suggesting that the association may be due to a high concentration of African genes in this population as compared to the general black population, rather than a direct interaction of G6PD deficiency with sickle cell disease. Both G6PD and hemoglobin S are selected for in the African mileu on the basis of an increased resistance to malaria. This has been shown quite elegantly for G6PD deficiency as a decreased infection rate of the G6PD-deficient erythrocytes as compared to the normal erythrocytes in the circulation of a female heterozygous for G6PD deficiency (27).

Theories of Drug-induced hemolysis

While the precise mechanism of drug-induced hemolysis in G6PD deficiency is not known, a general outline is currently accepted (2,22): potentially hemolytic drugs are converted by the host into active metabolites, which interact with oxyhemoglobin to form hydrogen peroxide or oxidative free radicals. Detoxification of hydrogen peroxide within the erythrocyte proceeds via glutathione peroxidase in the presence of reduced glutathione (Figure 5), although catalase may assist in detoxification when high levels of peroxide are present. Maintenance of reduced glutathione, however, depends on a continued supply of NADPH. Impaired G6PD activity in the G6PD-deficient erythrocyte hampers the detoxification effort by limiting the supply of NADPH. Cellu-

lar defenses are overwhelmed, and oxidative damage occurs to cellular constituents. Mixed disulfides are formed between glutathione and hemoglobin at the β93 cysteine residue, and are not reduced because of the unavailability of NADPH. This leads to conformational change of the hemoglobin molecule, with oxidation of interior sulfhydryl groups and irreversible denaturation. The denatured hemoglobin at this stage becomes visible as Heinz bodies when stained with methyl violet. Shortened erythrocyte survival results from splenic uptake of erythrocytes containing Heinz bodies. Coincident oxidative damage occurs to sulfhydryl containing enzymes, membrane sulfhydryl groups, and membrane lipid, perhaps contributing to erythrocyte destruction.

Biotransformation products of hemolytic drugs, rather than the drugs themselves, seem to be most active in causing hemolysis (13,39). Primaquine, for example, is no more toxic to G6PD-deficient erythrocytes than to normal erythrocytes in vitro, but urine or serum from primaquine-treated animals is selectively toxic to G6PD-deficient erythrocytes (39). The active metabolites of most drugs have not been identified, but Figure 7 shows the theoretically most active metabolites of several hemolytic drugs. They are each redox compounds capable of participating in the reaction shown in Figure 8 for the redox pair phenylhydroxylamine and its oxidized form, nitrosobenzene. According to the mechanism proposed by Kiese (26), the reduced form of the metabolite can undergo a coupled oxidation with oxyhemoglobin, to form methemoglobin and the oxidized form of the metabolite. Reversion to the reduced form of the metabolite then takes place through the action of NADPH dependent diaphorase. In brief, each molecule of drug metabolite acting by this mechanism can consume many molecules of erythrocytic NADPH, which is in short supply in the G6PD-deficient erythrocyte. Patterns of drug metabolism resulting in higher than usual levels of these active metabolites may be responsible for unusual individual sensitivity to drug-induced hemolysis, although this has not been confirmed.

Drug metabolites may interact directly with erythrocytic G6PD to cause hemolysis. A variety of known hemolytic drugs (13, 11) can cause competitive and non-competitive inhibition of G6PD

from yeast and normal erythrocytes. If variant enzymes are especially sensitive to inhibition by drugs, as some are to inhibition by normal red cell constituents (49,50), this phenomenon could contribute to drug-induced hemolysis. The interactions of variant enzymes with hemolytic drugs have not been studied sufficiently to resolve this question, but selective inhibition of G6PD Milwaukee by salicylate has been observed with G6PD from an individual who had undergone a hemolytic reaction to aspirin (34,40). On the other hand, stimulation rather than inhibition of the hexose monophosphate shunt has been observed in normal erythrocytes exposed to primaquine metabolites (44).

Whether the mode of injury is oxidative or inhibitory, the main effect is depletion of reduced glutathione. Depletion of glutathione in vitro has not been shown to damage the erythrocyte, unless a specific oxidative stress is imposed at the same time (22,25). Similarly, methemoglobin formation by nitrite administration or in congenital methemoglobinemia (NADH diaphorase deficiency) does not impair red cell survival (39,2). In fact nitrite induced methemoglobinemia does not enhance susceptibility to phenylhydrazine induced hemolysis (2), nor is methemoglobin necessarily seen in drug-induced hemolysis of G6PD-deficient erythrocytes. Moreover, splenic function does not seem to be necessary for drug-induced hemolysis: although acetylphenylhydrazine induced hemolysis proceeds less rapidly in splenectomized rats (1), it proceeds, and cases of drug-induced hemolysis in splenectomized G6PD-deficient humans have been reported (21). Perhaps it is a combination of damage to enzymes, membrane, and hemoglobin that is responsible for drug-induced hemolysis, but the exact chain of events is still unclear.

Currently Available Tests of Drug-induced Hemolysis

Detection of G6PD-deficient individuals is easy, but prediction of which drugs will induce hemolysis is difficult. Virtually all current information on this subject has been gained by *in vivo* testing. Hemolytic drugs have been administered to G6PD-deficient individuals (34) despite obvious risks to these individuals. A safer approach has been the transfusion of ^{51}Chromium labeled G6PD-deficient erythrocytes into compatible

human recipients who are treated with hemolytic drugs (12). It has not been applied to the large scale study of hemolytic drugs as they effect different G6PD variants, in part because of the risks to recipients. Thus most new information on drug-induced hemolysis comes from clinical reports, in which the precise variant of G6PD is not determined, and in which the effect of the underlying disease processes are not known.

Alternative methods of studying drug-induced hemolysis have been sought. They vary in the method of drug exposure (raw drug, predicted metabolites, or serum from drug-taking individuals), and in the endpoints used to evaluate drug effect (changes in erythrocyte morphology, biochemistry, or fragility). It is not known which metabolites are most active, nor which endpoints correlate best with *in vivo* hemolysis. Thus no currently available test is recognized as a reliable way of predicting drug-induced hemolysis. The most promising of these tests are reviewed below.

Acetylphenylhydrazine (29) and nitrofurantoin (24) have been shown to induce numerous, large Heinz bodies in G6PD-deficient erythrocytes. Other drugs have not produced similar effects.

Theoretical metabolites of primaquine and phenacetin can cause increased mechanical and osmotic fragility in both normal and G6PD-deficient erythrocytes *in vitro* (16,17). Other drugs have not been evaluated, and the correlation between mechanical fragility and *in vivo* hemolysis is not yet clear.

Derivatives of diaminodiphenylsulphone (Dapsone) have been used to challenge normal and G6PD-deficient red cells. Some stimulation of the hexose monophosphate shunt, fall in GSH level, and increase in auto-, osmotic-, and peroxide-induced hemolysis were observed (38), in both normal and G6PD-deficient cells.

Hexose monophosphate shunt activity of normal erythrocytes exposed to serum from their primaquine-taking hosts, has been measured by the rate of $^{14}CO_2$ evolution from glucose-1-^{14}C, (44). A small (one percent) increase in shunt activity was observed with the addition of primaquine-serum. This was boosted to a 14 percent increase by the addition of cyanide to both primaquine and control sera, perhaps by inhibition of catalase-mediated

detoxification of hydrogen peroxide. The significance of this small increase in shunt activity is unknown, since the hexose monophosphate shunt normally functions at only 0.1 to 0.2 percent of its capactiy (49), and should be able to withstand the small observed increments in even a G6PD-deficient cell. Other hemolytic drugs have not been tested

Animals have been sought that might have G6PD deficiency, so that hemolytic drugs could be administered. Sheep have been the most promising, with G6PD activities (Vmax) similar to those seen in G6PD-deficient humans. Exposure of 145 sheep to a variety of hemolytic drugs and fava beans caused no hemolysis (28). Perhaps their erythrocytic G6PD activity is normal despite the low measured Vmax, as has been reported for some human G6PD variants (49,50). Other animals with possible G6PD deficiency should be similarly tested, for a true animal model of drug-induced hemolysis would be invaluable.

PART II - Discussion of Experimental Results

Preliminary testing has shown that human G6PD-deficient erythrocytes are sensitive to drug-induced hemolysis by acetylphenylhydrazine, nitrofurantoin, and primaquine, when transfused into rat hosts pretreated with ethyl palmitate and cobra venom factor to prolong the survival of human erythrocytes. This model offers two main advantages over in vitro tests of hemolysis: 1)the rat host provides a continuous supply of drug metabolites, and 2) hemolysis is the measured endpoint. In these respects, the rat model resembles the human-to-human cross-transfusion experiments discussed above. A number of obvious differences are present: 1)the patterns of drug metabolism and excretion in rats may be very different from those in humans, 2) the partial reticuloendothelial blockade needed to allow erythrocyte survival may alter the hemolytic process, 3) the effects of antibody coating of the transfused erythrocyes, despite the blockage of complement-mediated hemolysis, are unknown, and 4) non-specific hemolysis of human erythrocytes in the rats limits the time available for the study of drug-induced hemolysis to 24 hours. Thus despite the similarities between hemolysis in the rat model and in the human, only

further testing can establish the ultimate validity of this model.

A number of interesting results were obtained during this study. Erythrocytes from the Mediterranean G6PD-deficient individual were nearly twice as sensitive to drug-induced hemolysis as were those from black G6PD-deficient subjects. This difference is compatible with the resistance of approximately the younger 50 percent of erythrocytes in black G6PD-deficient individuals to drug-induced hemolysis (30). This could be verified by harvesting and transfusing only the oldest and most G6PD-deficient erythrocytes from the black donors.

Somewhat disconcerting is the significant hemolysis seen with normal erythrocytes in drug-treated animals. Drug doses were intentionally large to force hemolysis, but this result suggests that a downward revision of drug dose may be in order. Preliminary studies have shown no loss of hemolytic effect with erythrocytes from the Mediterranean G6PD-deficient subject when the dose of nitrofurantoin was reduced from 30 mg/kg to 10 mg/kg.

Erythrocytes from two individuals showed hemolytic behavior different from other individuals in their groups. Black G6PD-deficient donor ST seemed relatively insensitive to the effects of acetylphenylhydrazine and nitrofurantoin, but particularly sensitive to primaquine. Conversely, normal donor PMcP seemed sensitive to both acetylphenylhydrazine and nitrofurantoin, but not to primaquine. In the second case, a wide spread between the survivals in the duplicate control rats (38 vs. 75 percent) raises the question of experimental artifact. Aberrant drug sensitivity, secondary to a G6PD sub-variant or other erythrocytic defect, needs to be investigated in these cases by repeat evaluations of the drug-sensitivities of these two individuals.

Results in these experiments suggest a number of avenues for further investigation. A larger number of individuals should be evaluated against a larger number of hemolytic drugs to establish the ultimate validity of the model, and to search for aberrant patterns of drug sensitivity. Perhaps this would be facilitated by the study of individuals who have experienced clinical hemolytic episodes with unusual hemolytic drugs such as chloramphenicol or aspirin. Precise dose-response evaluations of

hemolytic drugs should be executed to establish whether hemolytic effects persist when drug doses are reduced to the accepted human therapeutic ranges.

The potential applications of this model are several: 1) the screening of new drugs for hemolytic potential prior to clinical introduction, 2) the characterization of the hemolytic sensitivities of the 100 known G6PD variants to hemolytic agents in a systematic way, and 3) the screening of single patients for aberrant drug sensitivity, if this be mediated by intra-erythrocytic factors.

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TABLE 1

HEMATOLOGIC PARAMETERS OF HUMAN DONORS

DONOR	G6PD*	HEMOGLOBIN	HEMATOCRIT	RETICULOCYTES
CR_B**	7.7	AS	35%	-
CS_B	9.8	AA	41%	-
HD***	NL	-	-	-
PMP***	NL	-	-	-
MR***	NL	-	-	-
OC***	NL	-	-	-
DdL_M†	0.4	AA	41%	3.9%
KM_B	0.7	-	39%	1.5%
ST_B	0.5	AA	39%	-
FW_B	1.0	AS	46%	-

*G6PD activity expressed in international units, equal to micromoles of substrate converted per minute per gm hemoglobin at 30°C. Normal range 6-10 IU, low activity 0-4 IU

**Subscripts indicate racial origin: B=Black
M=Mediterranean

***These individuals are members of the Department of Hematology, Yale-New Haven Hospital, whose parameters are known to be normal, and are not reported here

†DdL is a 26 year old male of Italian parentage who presented with a severe hemolytic episode during recovery from an appendectomy at age 14. Hemolysis abated when all drugs were discontinued; tentative hemolytic agent was aspirin. Since that time he has been in good general health except for occasional episodes of dark urine following ingestion of analgesics, slight scleral icterus, and a mild compensated non-spherocytic hemolytic anemia.

TABLE 2

THE 24-HOUR SURVIVAL (IN PERCENT) OF HUMAN ERYTHROCYTES IN RAT HOSTS*

DONOR	5% DEXTROSE	APH	FURA	PRIMA
CR_{B}**	55± 4	47± 8	54± 6	41±12
CS_{B}	51± 6	49±13	55± 1	45± 6
HD	58±15	55± 2	52± 5	52± 3
PMP	57±26	21± 1	37±17	47±10
MR	79± 8	72±11	72± 3	66± 8
OC	47± 1	42± 5	44±13	36± 2
MEAN(6 NORMAL)	58±13	47±16	52±13	48±12
DdL_{M}	57±12	18± 2	15± 2	20±10
KM_{B}	56±12	32± 3	42± 1	39± 4
ST_{B}	63±14	54±28	59± 4	18± 1
FW_{B}	56± 1	31±12	46± 1	33± 1
MEAN(4 DEFIC.)	58± 3	34±15	40±18	27± 9

*Each figure is the mean ± standard deviation in duplicate rats

**Subscripts indicate racial origin: B= Black
M= Mediterranean

TABLE 3

DRUG-INDUCED HEMOLYSIS (IN PERCENT) 24 HOURS AFTER TRANSFUSION*

DONOR	APH	FURA	PRIMA
CR_B	14	2	25
CS_B	4	-4	12
HD	5	10	10
PMP	63	35	18
MR	9	9	16
OC	9	5	22
MEAN(6 NORMALS)	17%	10%	17%
DdL_M	68	74	70
KM_B	43	25	30
ST_B	14	6	71
FW_B	45	18	41
MEAN(4 DEFICIENTS)	42%	31%	53%

*Drug-induced hemolysis calculated from 24-hour survival data in Table 2 as:

$$100\% \text{ minus } \left(\frac{\text{Survival (drug)}}{\text{Survival (dextrose)}}\right) \times 100$$

FIGURE 1

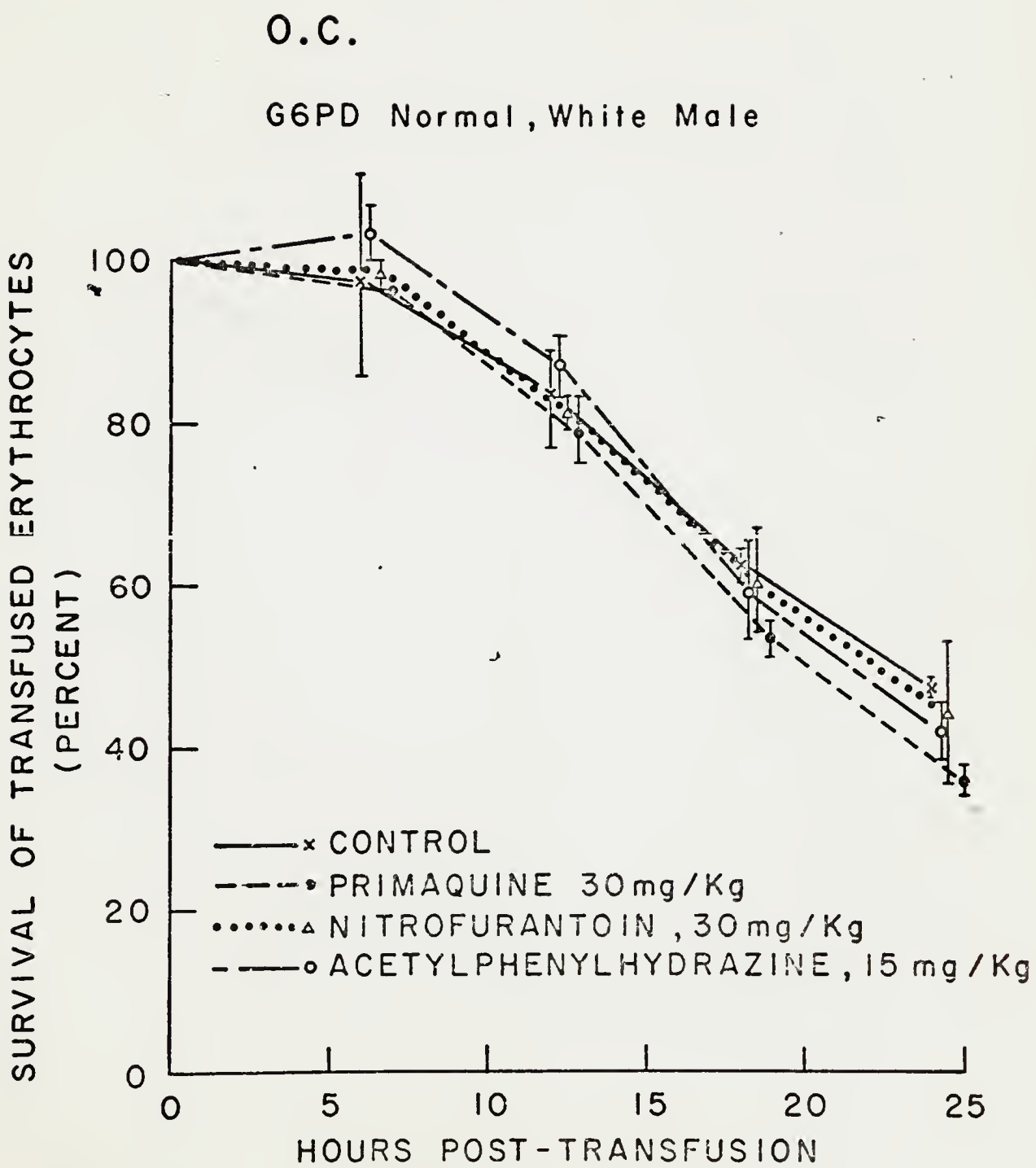

O.C.
G6PD Normal, White Male
SURVIVAL OF TRANSFUSED ERYTHROCYTES (PERCENT)
100
80
60
40
20
0
0
5
10
15
20
25
HOURS POST-TRANSFUSION
CONTROL
PRIMAQUINE 30mg/Kg
NITROFURANTOIN, 30mg/Kg
ACETYLPHENYLHYDRAZINE, 15 mg/Kg

FIGURE 2

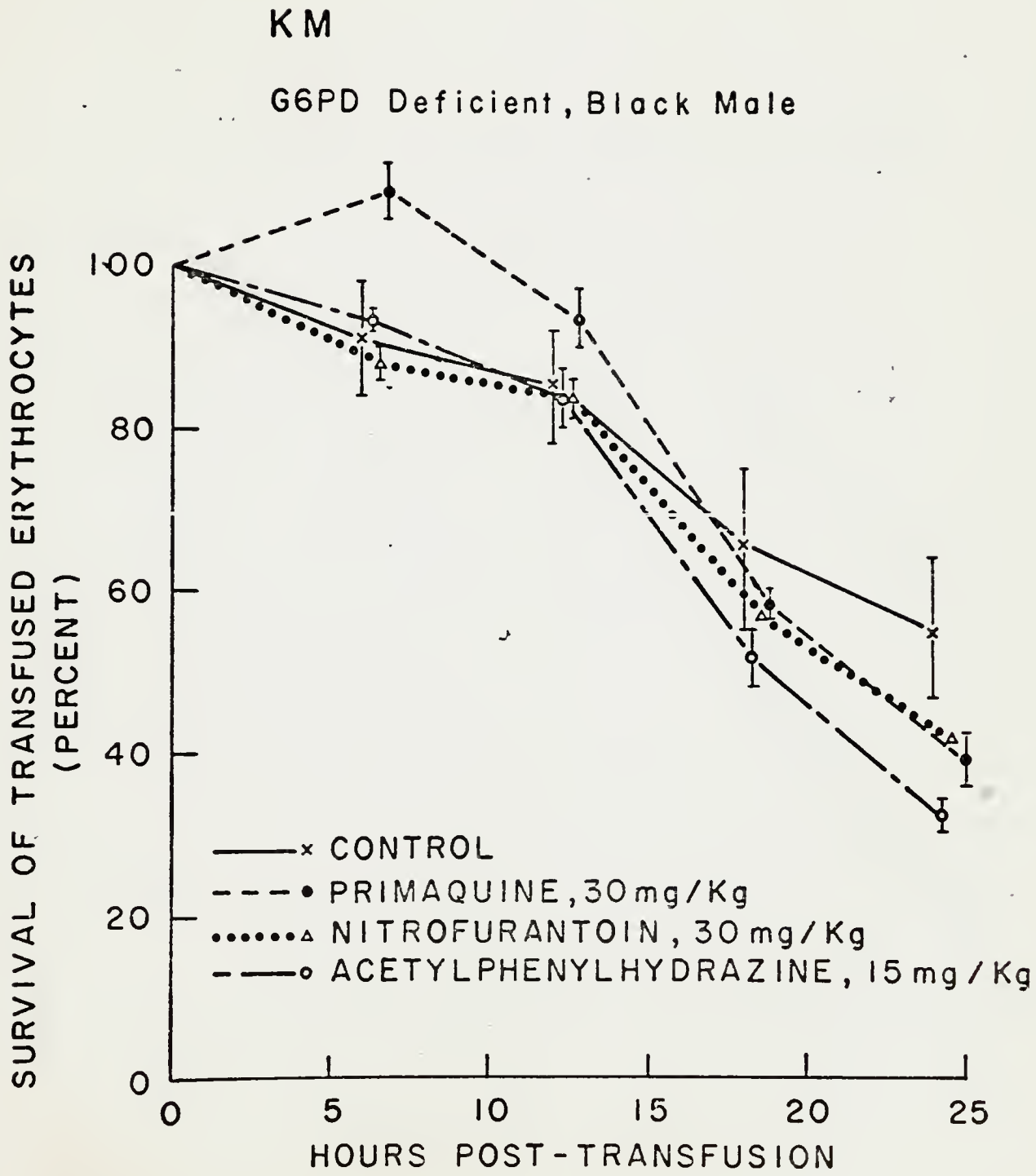

KM
G6PD Deficient, Black Male
SURVIVAL OF TRANSFUSED ERYTHROCYTES (PERCENT)
100
80
60
40
20
0
0
5
10
15
20
25
HOURS POST-TRANSFUSION
CONTROL
PRIMAQUINE, 30 mg/Kg
NITROFURANTOIN, 30 mg/Kg
ACETYLPHENYLHYDRAZINE, 15 mg/Kg

FIGURE 3

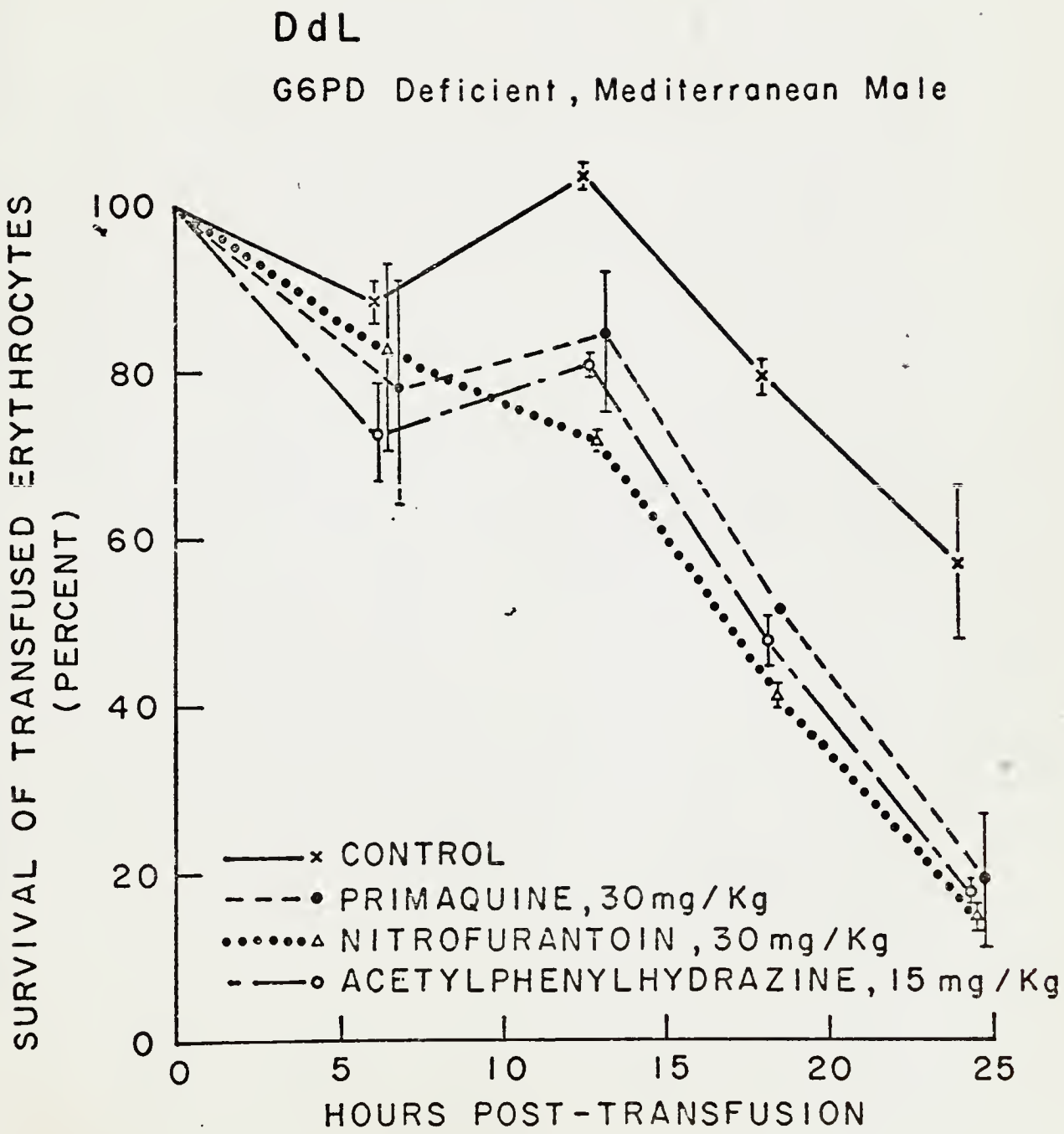

DdL
G6PD Deficient, Mediterranean Male
SURVIVAL OF TRANSFUSED ERYTHROCYTES (PERCENT)
100
80
60
40
20
0
0
5
10
15
20
25
HOURS POST-TRANSFUSION
CONTROL
PRIMAQUINE, 30mg/Kg
NITROFURANTOIN, 30mg/Kg
ACETYLPHENYLHYDRAZINE, 15mg/Kg

FIGURE 4

Each point represents one human subject tested against the indicated drug (see Key). The mean percentage drug-induced hemolysis of the six normal individuals is shown by a solid arrow, and that of the G6PD-deficient individuals by an open arrow, for each of the three drugs tested.

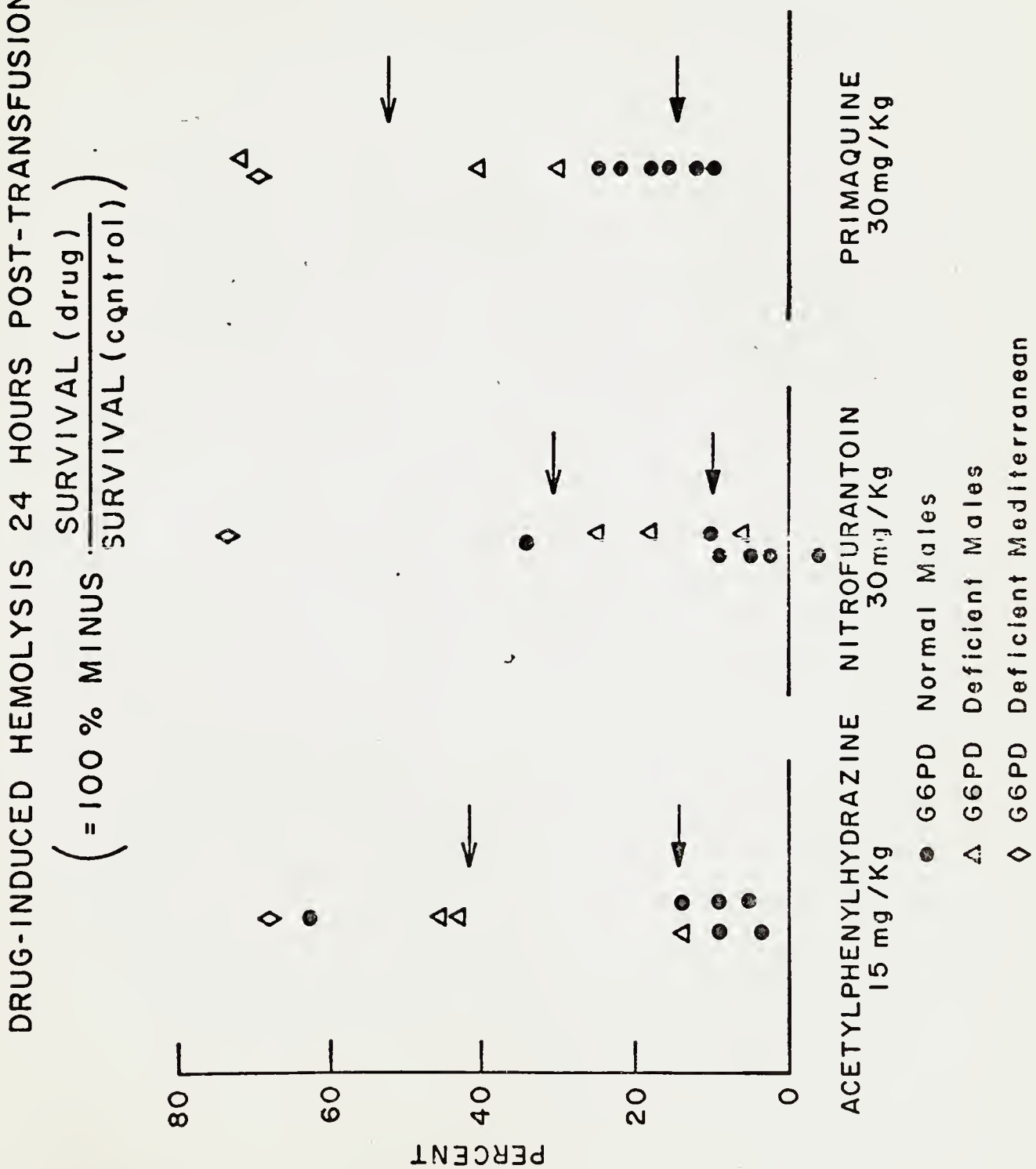


FIGURE 5

HEXOSE MONOPHOSPHATE SHUNT (reference 2)
and red cell glucose metabolism

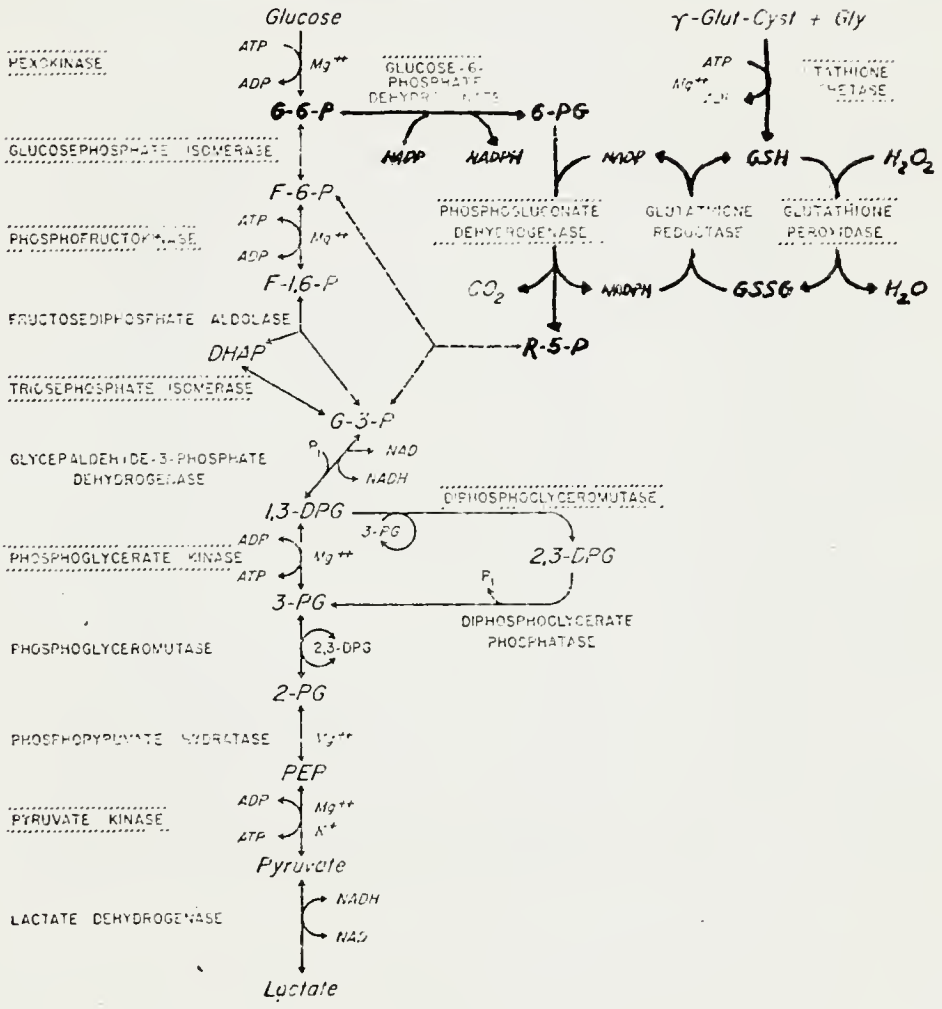


FIGURE 6 (from Miale, reference 29)

Arrows indicate the drugs used in these experiments. A more extensive list of drugs known to cause hemolysis in G6PD-deficient individuals can be found with documentation in the references (2,45)

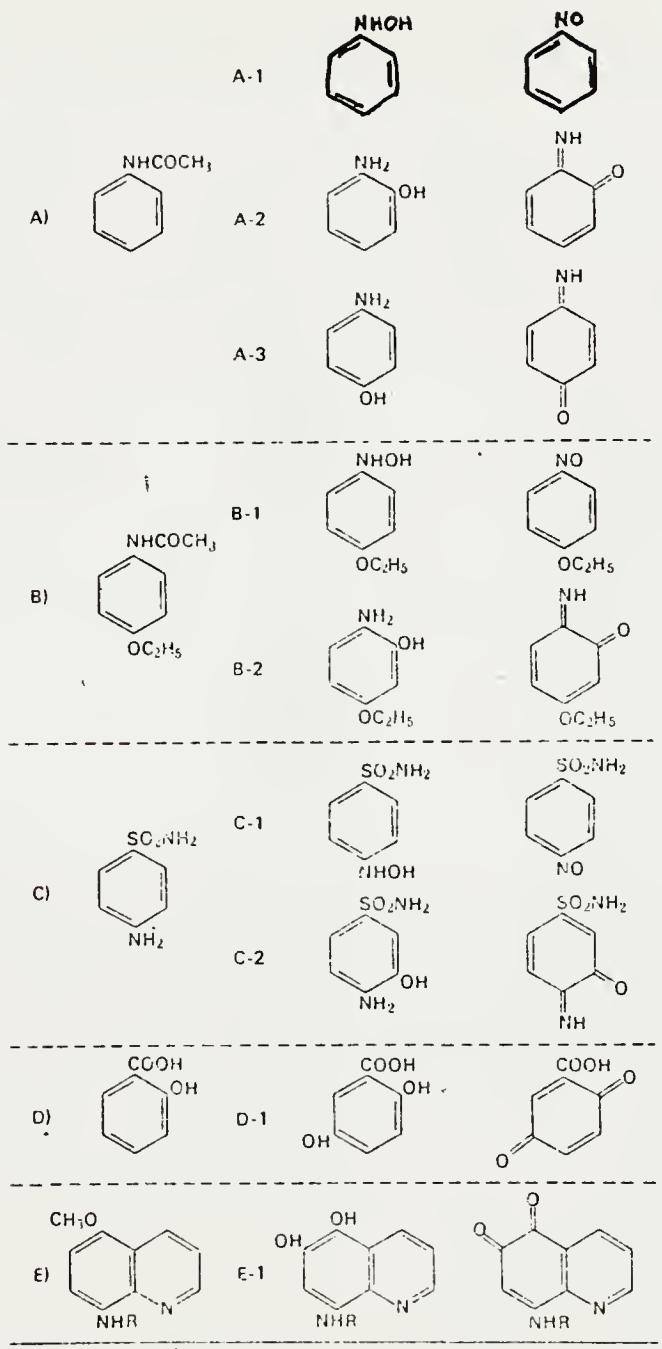
Drugs and other agents that can induce in vivo hemolysis of G-6-PD-deficient erythrocytes*

Group I: Antimalarials	Furazolidone
→ Primaquine	Furaltadone
Pamaquine	Sulfoxone
Pentaquine	Probenecid
Plasmoquine	→ Acetylphenylhydrazine
Quinocide	Naphthalene
Group II: Sulfonamides	Methylene blue
Sulfanilamide	Vitamin K (water-soluble derivatives)
Sulfapyridine	Chloramphenicol
Sulfisoxazole	Thiazolsulfone
Salicylazosulfapyridine	Diaminodiphenylsulfone
Sulfamethoxypyridazine	Trinitrotoluene
Sulfacetamide	Quinidine
Group III. Salicylates and analgesics	Neosalvarsan
Aspirin	Group V: Infectious and metabolic disorders
Acetanilid	Viral respiratory disease
Acetophenetidin	Viral hepatitis
Antipyrine	Infectious mononucleosis
Aminopyrine	Bacterial sepsis
p-Aminosalicylic acid	Diabetic acidosis
Group IV: Other drugs	Renal failure
→ Nitrofurantoin	Group VI: Special agent
Nitrofurazone	Fava bean

*In most instances the hemolytic effect of the drug is dose related. In some instances the hemolytic effect is the result of drug intake plus predisposing conditions such as infection.

FIGURE 7 (from Shahidi, reference 39)

"Hemolytic" drugs and their "oxidant" metabolite



A) Acetanilide A-1) Phenylhydroxylamine A-2) o-Aminophenol A-3) p-Aminophenol.

B) Acetophenetidin. B-1) p-Ethoxyphenylhydroxylamine C-2) 3-Hydroxyphenetidin.

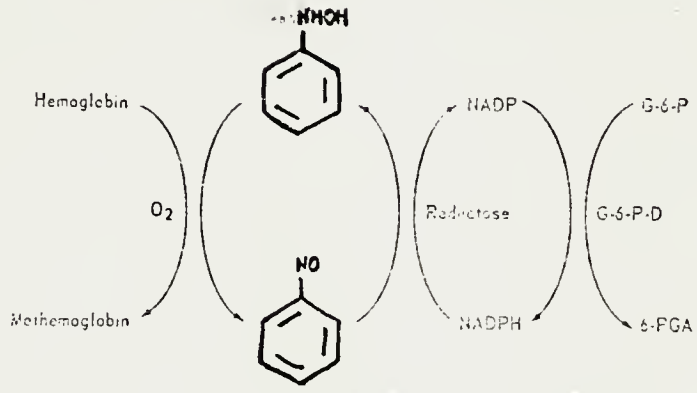
C) Sulfanilamide. C-1) p-Hydroxylaminobenzosulfonamide C-2) 3-Hydroxysulfanilamide.

D) Salicylic acid. D-1) Gentisic acid.

E) 6-Methoxyaminoquinolone derivative. E-1) 5,6-Quinoline quinone.

FIGURE 8

COUPLED OXIDATION PATHWAY (OF KIESE, reference 26)



The reduced form of the drug (phenylhydroxylamine) undergoes coupled oxidation with oxyhemoglobin, to form methemoglobin and the oxidized form of the drug (nitrosobenzene). The latter is re-reduced to its original form by NADPH, and the cycle is repeated.

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